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12 PERSONAL AUTHOR(S)

Sikes, C. Steven, Wheeler, A.P., and Little, Brenda J. (B.J.L., NORDA, NSTL, MS)

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In the first year of the project, the work has been focused on 1) synthesis of polypeptides, 2) evaluation of their activities as inhibitors of calcium carbonate and phosphate crystallization, and 3) measurement of their activities as inhibitors of corrosion of mild steel. A set of polyaspartate molecules of specific molecular sizes and other aspartate-enriched peptides were made by automated solid phase methods. Polyaspartate molecules with 1 to 3 serine residues at the N-terminus were phosphorylated post-synthesis using monochlorophosphoric acid. The polyaspartate molecules were very effective inhibitors of calcium carbonate but not calcium phosphate crystallization. Polyaspartate was most effective as an inhibitor of $CaCO_3$ crystal growth at a length of about 15 residues, but a length of about 35 residues was required for maximum inhibition of crystal nucleation.

The polyaspartate molecules with terminal phosphoserine residues were extra-

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ordinarily active inhibitors of both calcium carbonate and phosphate formation. A thermal method of synthesis of polyaspartate based on peptide bond formation in dry powders of aspartic acid at around 200°C was developed to produce larger sample sizes required in corrosion experiments. Bulk thermal polyaspartate at 0.1 mg/ml ennobled the corrosion potential of mild steel electrodes somewhat with a reduction in corrosion rate from about 7 to 2 milli-inches per year. By use of scanning electron microscopy, the polyaspartate was seen to be bound to electrode surfaces after brief exposures, where it appeared to inhibit both anodic and cathodic reactions at doses used in practice for corrosion inhibition.

DATE: 1 July 1989

ANNUAL REPORT

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PRINCIPAL INVESTIGATOR: C. Steven Sikes

CO-INVESTIGATORS: A.P. Wheeler and Brenda J. Little

CONTRACTOR: The University of South Alabama

CONTRACT TITLE: Polypeptide Inhibitors of Mineral Scaling and Corrosion

START DATE: 1 July 1988

RESEARCH OBJECTIVES:

The overall purpose of the project is to understand better the interactions of peptide inhibitors of mineral scaling and corrosion with metal surfaces, including relationships between mineral deposition and corrosion. The peptides are based on natural protein inhibitors of mineral formation and generally are enriched in aspartic acid and phosphoserine. Specifically, the project involves 1) synthesis of polypeptides, 2) evaluation of their activities as inhibitors of calcium carbonate and phosphate crystallization, and 3) evaluation of their activities as inhibitors of corrosion of mild steel.

PROGRESS (YEAR 1):

Synthesis of Polypeptides.

Automated solid-phase synthesis. A peptide synthesizer (Applied Biosystems, Model 430A) was used to prepare peptides. A family of polyaspartate molecules ranging in size from Asp_5 to Asp_{60} was synthesized by repetitive couplings of t-Boc-L-aspartic acid residues with B-carboxyl protection by O-benzyl linkage. A C-terminal residue of 0.5 mmole was preloaded on a resin of a polymer of styrene cross-linked with 1% of divinylbenzene. The C-terminal amino acid was linked to the resin via a phenylacetamidomethyl (PAM) group. Peptide bond formation was promoted by use of dicyclohexyl carbodiimide (DCC) and formation of symmetric anhydrides of the incoming amino acid. At the appropriate times during a synthesis, a subsample of the peptide-resin was taken to provide the desired size of the polyaspartate, then the synthesis was allowed to continue to produce the next larger size, and so forth until all desired sizes had been synthesized and collected.

The same procedure was followed in synthesizing a family of $aspartate_{15}$ $alanine_x$ molecules ranging from $Asp_{15}Ala_2$ to $Asp_{15}Ala_{10}$. In this case, t-Boc-L-alanine residues were used. For peptides containing serine or glycine residues, t-Boc-L-serine-O-benzyl and t-Boc-L-glycine were used.

In all cases, coupling efficiency of each residue was checked by automated sampling of peptide resin for measurement of unreacted free amine by the ninhydrin method. Coupling efficiencies routinely were greater than 99% per cycle of synthesis.

Following synthesis, peptide-resin was repeatedly washed with methanol then dried and weighed. Then peptides were cleaved from the resin using a modification of the trifluoromethyl sulfonic acid (TFMSA) procedure, with precautions taken to prevent aspartimide formation. For 100 mg samples, peptide-resins in a scintillation vial were treated for 10 minutes with 150ul of anisole to swell the resin, making it more accessible for reaction. Then 1.0 ml of neat trifluoroacetic acid (TFA) was added with magnetic stirring and allowed to react for 10 minutes. Next, 100 ul of concentrated TFMSA (Aldrich Chemical Co.) were added with cooling using an ice bath, followed by cleavage of the peptide from the resin at room temperature for 30 minutes. For cleavage of other amounts of peptide-resin, the amounts of reagents used were changed proportionally.

Following cleavage, 20 ml of methyl butyl ether (MBE) (Aldrich) were added to the vial to insure precipitation of the peptide, which already was relatively insoluble in the acidic reaction medium due to the acidic nature of the peptides. After stirring for 1-2 minutes, the entire slurry was passed through a 4.25 cm glass fiber filter (Fisher G4) using a filter funnel and vacuum pump at 15 psi. This removed the TFA, TFMSA, anisole, and any soluble reaction products, leaving the cleaved peptide and resin on the filter. After washing on the filter with 100 ml of MBE, the peptide was extracted into a clean, dry flask with 10 ml of Na_2CO_3 (0.02 M, pH 10.2), using 5 successive rinses of 2 ml, with at least 1 minute extraction on the filter prior to applying the vacuum each time. Using this procedure, the filtrate containing the solubilized peptides had pH values >5. The filtrate was then dialyzed twice with stirring against 2 liters of distilled water for 2 hours using dialysis tubing (Spectrapor, nominal MW cutoff of 1000 daltons). The dialysate was frozen and lyophilized, yielding white flakes or powders. The average yield of the peptides was 40%.

Following isolation, purity of the peptides was checked by high performance liquid chromatography (Varian 5500 LC) using gel permeation columns designed for separations of peptides (Toya Soda 2000 SW and 3000 PWXL). Single, sharp peaks at the appropriate MW were obtained. Because the peptides were isolated partially as sodium salts, the sodium content was determined by atomic absorption (Perkin Elmer model 360). Sodium levels typically were less than 5% by weight. Amounts of peptides reported were corrected for sodium content. Concentrations of peptides in aqueous stock solution were based on lyophilized dry weight but were also checked by comparison of UV spectra.

Post-synthesis phosphorylation. Because phosphate linkages to proteins are not stable during cleavage steps of solid-phase synthesis and because phosphate is known to be critical to the activity of the protein inhibitors of mineral formation, we evaluated several methods of preparation of phosphopeptides. These included direct polymerization of

phosphoserine by use of DCC, and phosphorylation of polyserine by use of diphenyl phosphochloridate followed by catalytic hydrogenolysis to remove phenyl protecting groups. Both of these approaches gave unsatisfactory results. However, a successful method based on the use of monochlorophosphoric acid was employed, with production of peptides with essentially 100% phosphorylation of serine residues.

In this approach, serine residues attached at the N-terminus of polyaspartate molecules were phosphorylated manually using the following procedure and in proportions consistent with the molar amounts of serine residues. For example, phosphorus oxychloride, $POCl_3$, was added as 117 ml (1.25 moles) to 45 ml (2.5 moles) of water. This solution was stirred for one hour, allowing formation of monochlorophosphate (ClH_2PO_3). Next, an amount of peptide to provide 0.25 moles of serine residues was added with stirring and occasional heating at 60°C for two hours. The reaction was ended by dropwise addition of 18 ml (1 mole) of H_2O to degrade any unreacted monochlorophosphate to orthophosphate. Any polyphosphates that may have formed during the reaction were destroyed by addition of 75 ml of 1N HCL and heating in a boiling water bath for at least 20 minutes. Upon cooling, peptides were crystallized in 95 percent ethanol and methyl butyl ether at 3°C overnight. Crystals were washed repeatedly with ethanol. The extent of phosphorylation of peptides was monitored spectrophotometrically upon formation of the phosphomolybdate complex.

Thermal polymerization. Because the measurements of activity of the peptides as corrosion inhibitors, as well as other procedures, including pilot-scale studies in some cases, require relatively large amounts of materials (from 1 gram on up) and because available solid-phase synthesis becomes prohibitively expensive at that level, we looked into the feasibility of bulk thermal polymerization of aspartic-enriched polyamino acids. This approach to polypeptide synthesis originated in the study of the possible abiotic origin of proteins.

In a typical synthesis, L-aspartic acid (500 g) was placed in a two-liter, round-bottom reaction vessel, originally designed as the evaporator vessel in a rotary evaporator apparatus. The reaction vessel was partially submerged in cooking oil in a deep-fryer set at 190°C/ ($\pm$2°C). The reaction vessel was coupled by ground glass fitting to a condenser vessel, which in turn was fitted to a rotator shaft driven by a rheostated electric motor. The fittings were sealed with tape and fastened with hose clamps. A stream of nitrogen was continuously purged into the condenser vessel to eliminate O_2 and the possibility of charring. The reaction was allowed to continue for up to 24 hours at which time no further visible evolution of water vapor was observed. The water is produced as a result of the dehydration reaction of peptide bond formation and serves as a good indicator of the progress of the reaction.

Polyaspartate molecules of approximately 5000 daltons (determined by gel permeation) were produced. They were purified by dissolving at pH 6 in water followed by dialysis to remove unreacted aspartic acid, although the bulk product is also usable without further purification.

Samples as large as 1 kilogram were prepared essentially at the cost of the amino acids (e.g., Sigma L-aspartic acid, $25.70/kg). A number of interesting materials were produced.

Assays to verify the identity of each of the molecules produced by both solid-phase and thermal methods included amino acid analysis, peptide sequencing studies, amine measurement by alkalimetric titration and ninhydrin reaction, carboxyl measurement by alkalimetric titration, phosphate analysis, and ultraviolet and infrared spectroscopy.

Evaluation of Polypeptides Enriched in Aspartic Acid and Phosphoserine as Inhibitors of Crystallization.

A number of assays have been developed to measure the ability of the peptides to inhibit mineral formation. These include methods for assessing effects on both crystal nucleation and crystal growth. The general results were that at a molecular size of about 15 residues, polyaspartate was most effective as an inhibitor of $CaCO_3$ crystal growth. However, the optimum size for inhibition of $CaCO_3$ crystal nucleation by polyaspartate was about 35 residues. In addition, the presence of an hydrophobic domain of alanine residues attached at the N-terminus of polyaspartate molecules of 15 residues enhanced the effectiveness of the molecules as inhibitors of crystal nucleation relative to control polyaspartates of appropriate sizes. This type of data is useful in formulating hypotheses about the nature of the crystal nuclei and the crystal growth sites and in designing effective inhibitors.

Perhaps the most interesting discovery of the past year was the remarkable positive effect that even a single phosphoserine residue can have on the activity of a polyaspartate as an inhibitor of crystallization. For example, as shown in Figure 1, the polyaspartate molecules that had one to three phosphoserine residues on the end were far more effective inhibitors of $CaCO_3$ formation as indicated by the length of time prior to crystallization, shown by stabilization of pH of the metastable solution at about pH 8.3. Note also that the most effective molecule measured was HO-$(Asp)_{20}$-$(pSer)_1$-H followed by HO-$(Asp)_{20}$-$(pSer)_3$ and HO-$(Asp)_{20}$-$(pSer)_2$-H. Thus, surprisingly, a single phosphoserine residue at the end of the molecule was the best arrangement, and adding more phosphoserines diminished the activity of the molecules.

Similarly, as seen in Figure 2, the polyaspartate molecules having one to three phosphoserine residues on the end were by far the most effective as inhibitors of calcium phosphate formation. In these experiments, HO-$(Asp)_{20}$-$(pSer)_2$-H was clearly the most effective molecule, followed by HO-$(Asp)_{20}$-$(pSer)_1$-H, with HO-$(Asp)_{20}$-$(pSer)_3$-H having much less activity, although still considerably more than simple HO-$(Asp)_{20}$-H or HO-$(Asp)_{25}$-H molecules. However, as shown in Table 1, the serine residues of the HO-$(Asp)_{20}$-$(pSer)_3$-H molecules were incompletely phosphorylated due to limitations of the method of phosphorylation. In any event, it is clear that having one or two phosphoserine residues at the end of a polyanionic peptide greatly enhances inhibitory activity, and in all cases the presence of phosphoserine improved the performance of peptides as inhibitors of crystallization. Furthermore, the polyaspartate-phosphoserine molecules are among the best known inhibitors of both calcium carbonate and calcium phosphate crystallization. This breadth of activity is itself unusual.

Figure 1. Effects of Phosphorylation of Synthetic, Polyanionic Peptides on Calcium Carbonate Crystallization.

In all cases, the concentration of peptides was 0.05 ug/ml.

In these experiments, a solution supersaturated with respect to $CaCO_3$ is prepared by separately pipetting 0.3 ml of 1.0 M $CaCl_2$ dihydrate and 0.6 ml of 0.4 M $NaHCO_3$ into 29.1 ml of artificial seawater (0.5 NaCl, 0.011 M KCl). Inhibitors normally are added after the calcium but before the bicarbonate. The reaction vessel is a 50 ml, 3-necked, round-bottom flask partially immersed in a thermostated water bath at 20°C. The reaction vessel is closed to the atmosphere to minimize exchange of CO_2. The reaction is started by adjusting the pH upward to 8.3 by titration of ul amounts of 1 N NaOH by digital pipette. The initial concentrations are 10 mM of Ca^{2+} and 8 mM of dissolved inorganic carbon (DIC). The reaction is monitored by pH electrode and recorded by strip chart. After a period of stable pH during which crystal nuclei form, the pH begins to drift downward until the reaction ceases due to depletion of reactants and the lowering of pH.

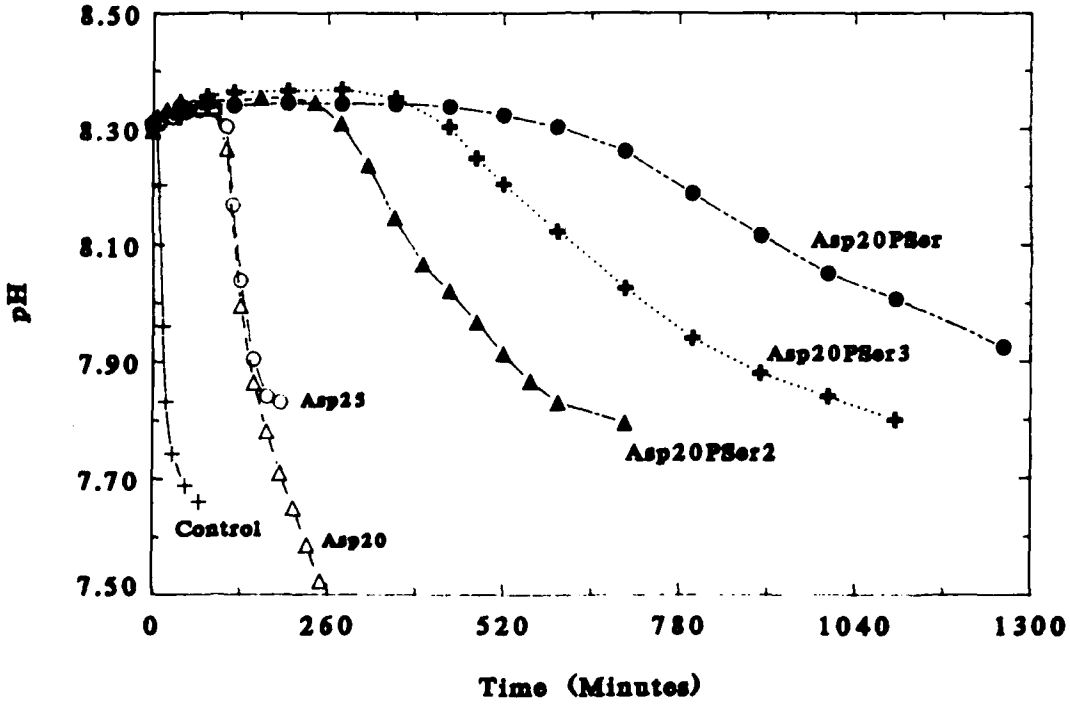


Figure 2. Effects of Phosphorylation of Synthetic, Polyanionic Peptides on Calcium Phosphate Crystallization.

In all cases, the concentration of the peptides was 30 ug/ml.

A solution supersaturated with respect to calcium phosphate is prepared by separately pipetting 0.1 ml of 1.32 M $CaCl_2$ dihydrate and 0.1 ml of 0.90 M NaH_2PO_4 into 29.8 ml of distilled water. This yields initial concentrations of 4.4 mM Ca^{2+} and 3.0 mM dissolved inorganic phosphorus (DIP). Inhibitors normally are added after the calcium but before the phosphate. The reaction vessel is a 50 ml, round-bottom, 3-necked flask partially immersed in a thermostated water bath at 20°C. The reaction vessel is closed to the atmosphere. The reaction begins upon mixing the reactants with an initial pH of 7.4.

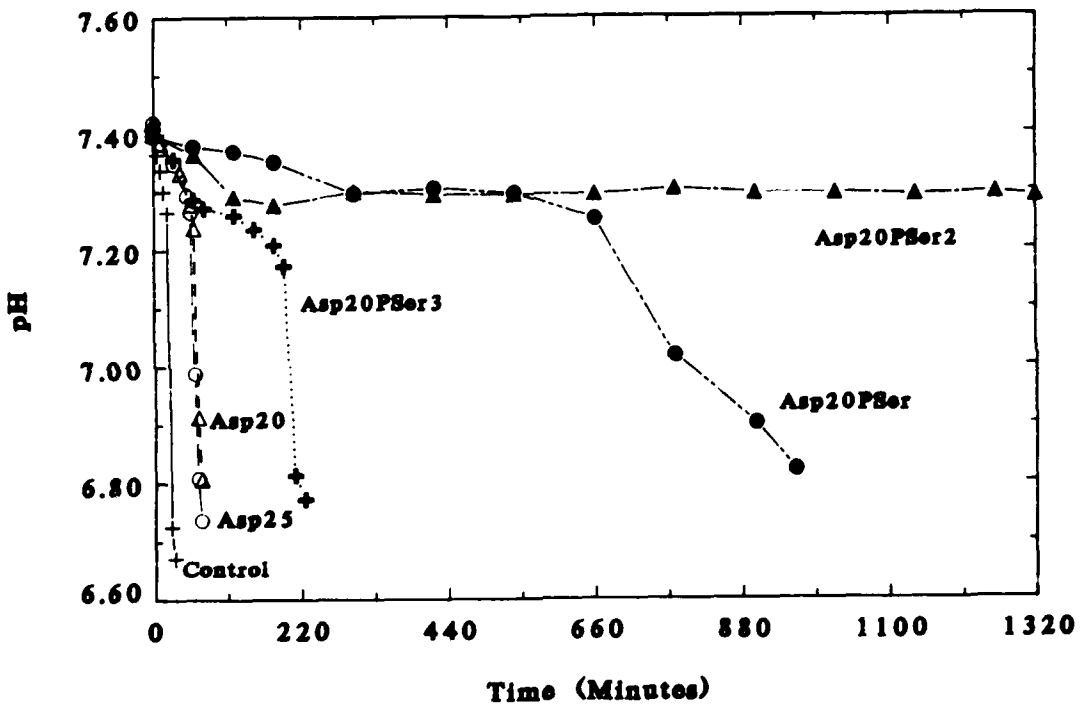


Table 1. Comparison of phosphorylated and non-phosphorylated serine-containing peptides as inhibitors of calcium carbonate and phosphate formation.

Peptide	%Ser as PSer	Calcium Carbonate Assay[a] (ug/ml)	Induction Period (minutes)	Calcium Phosphate Assay[b] (ug/ml)	Period to Apatite formation (minutes)
Control			5.86 ± 0.76[c]		20.7 ± 2.09[c]
PolySer(mw5100)		0.05	4.00 ± 0.28	10	same as control
PolyPSer	23.0	0.05	113 ± 50.6	10	33.5 ± 8.74
$(AspSerGly)_{10}$		0.10	9.10 ± 1.20	10	same as control
$(AspPSerGly)_{10}$	30.0	0.10	7.50 ± 2.10	10	28.0 ± 1.70
$(AspSer)_{10}$		0.05	33.5 ± 2.12	10	same as control
$(AspPSer)_{10}$	29.0	0.05	205 ± 14.4	10	31.0 ± 2.80
Asp_{20}		0.05	87.5 ± 19.0	30	59.7 ± 6.40
$Asp_{20}Ser$		0.05	65.0 ± 11.1	30	47.3 ± 3.05
$Asp_{20}PSer$	97.6	0.05	583 ± 5.80	30	601 ± 103
$Asp_{20}Ser_2$		0.05	48.8 ± 16.2	30	54.5 ± 3.50
$Asp_{20}PSer_2$	99.8	0.05	254 ± 39.9	30	> 20 hours
$Asp_{20}Ser_3$		0.05	31.3 ± 11.1	30	53.0 ± 0.75
$Asp_{20}PSer_3$	59.0	0.05	326 ± 88.0	30	185 ± 9.30

a. $CaCO_3$ pH-drift assay: 10mM Ca^{2+}, 8mM dissolved inorganic carbon, 30ml artificial seawater, 20°C., initial pH = 8.30.

b. $CaPO_4$ pH-drift assay: 4.4mM Ca^{2+}, 3mM dissolved inorganic phosphorus, 30 ml distilled water, 20°C., initial pH = 7.40.

c. Mean values ± standard deviations, n = 3 to 70.

Evaluation of Thermal Polyaspartate as a Corrosion Inhibitor.

A sample of polyaspartate prepared by the thermal polycondensation reaction was chosen for the initial measurements of corrosion inhibition. Because a single experiment of this type may require up to 1 gram of material, and we had 100's of grams of thermal polyaspartate, it was sensible to use this material in these studies. In addition, the results for a generic polyaspartate can serve as a basis for comparison to results for other molecules.

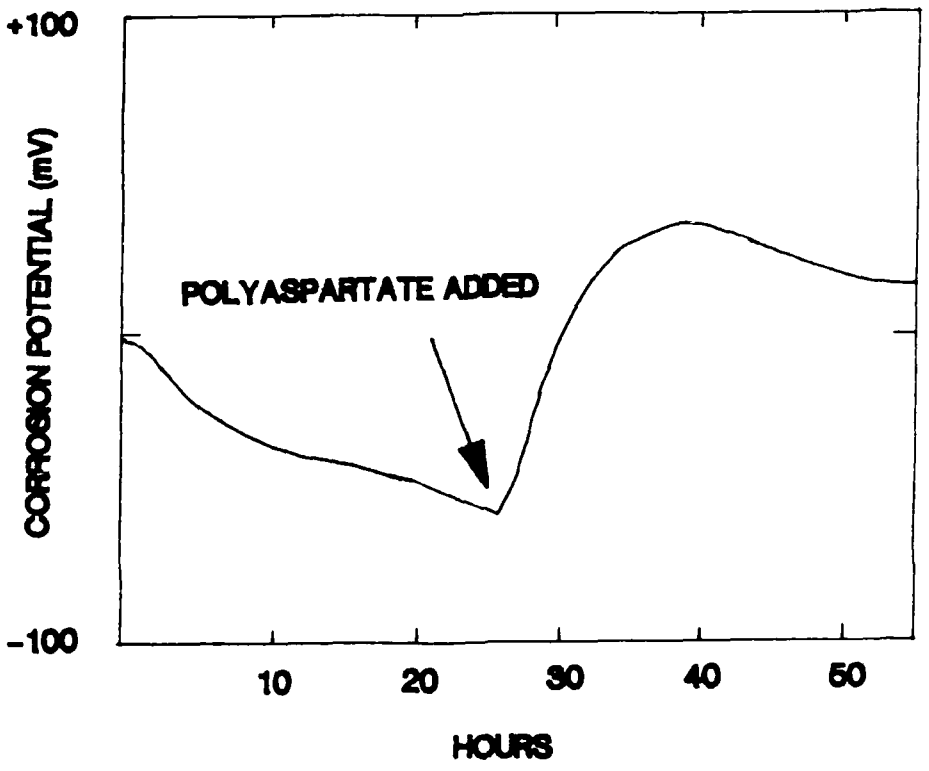
At a dose of 0.1 mg/ml of bulk thermal polyaspartate, which for this sample was equivalent to about 40 ug/ml of pure thermal polyaspartate, the corrosion potential of mild steel electrodes was ennobled somewhat (Figure 3). This was accompanied by a reduction in corrosion current from about 17 to 5 microamps per cm^2, which translated to a reduction in corrosion rate from about 7 to 2 milliinches per year.

Scanning electron microscopy confirmed that the polyaspartate was bound to the surface of the electrode after brief exposures. Analysis by EDAX showed some calcium and chlorine associated with the polyaspartate.

The results to date demonstrate that the material is surface-reactive and appears to inhibit both anodic and cathodic reactions of mild steel surfaces at doses of polymers that are used in practice for corrosion inhibition.

Figure 3. The effect of thermal polyaspartate on the corrosion potential of a mild steel electrode.

The concentration of bulk thermal polyaspartate was 0.1 mg/ml in artificial seawater. Potentials were measured relative to a saturated calomel electrode.



Work Plan (Year 2):

A main objective is to evaluate the phosphopeptides as corrosion inhibitors. To do this, we will first have to prepare larger amounts of the materials by thermal polymerization, followed by derivatization. Because serine residues do not survive thermal synthesis, asparagine residues, which are thermally stable, will be added to provide a reactive NH_2 group as a site of phosphorylation or phosphonation.

We recently received support from the State of Alabama to purchase a corrosion monitoring system for the laboratory at South Alabama. With this instrument, we will be able to screen the corrosion inhibiting activity of experimental compounds, and then select the most promising for more detailed mechanistic studies by Dr. Little at the Naval Space Technology Laboratory.

We also plan to synthesize sulfopeptides by methods analogous to those for preparing phosphopeptides. The literature suggests that sulfate or sulfonate containing polymers may be very effective inhibitors.

A general goal of the project is to understand more about the relationships between mineral deposition and corrosion, including microbially-accelerated corrosion. To begin a study of these relationships, experiments are planned in which supersaturated, spontaneously mineralizing solutions are used with and without the presence of the peptides, which inhibit both mineral deposition and corrosion.

Similarly, experiments are scheduled in which corrosion-accelerating microbes will be present in the experimental solution, including calcifying bacteria in some cases. This will allow us to begin exploring the relationship between microbially-induced corrosion and mineral deposition.

Inventions (Year 1):

A United States patent application, "Inhibition of mineral deposition by phosphorylated and related polyanionic peptides" (serial #07/334,456), by C. Steven Sikes was filed on April 7, 1989. This application covers the materials themselves and their use as inhibitors of crystallization. As we develop the data on corrosion inhibition, we will need to prepare an application in that field of use.

Publications and Reports (Year 1):

We organized and hosted a symposium, "Surface reactive peptides and polymers," that was held in April 1989 in Dallas as part of the 197th national meeting of the American Chemical Society. Our work was presented along with 26 other papers, scheduled for publication as an ACS book. The booklet of abstracts is enclosed.

1. Little, Brenda J. and C. Steven Sikes. 1989. Corrosion inhibition by thermal polyaspartate. Abstract, ACS 197th annual meeting.

2. Sikes, C. Steven and M.L. Yeung. 1989. Peptides enriched in aspartate and phosphoserine as inhibitors of calcium carbonate and phosphate crystallization. Abstract, ACS 197th annual meeting.

3. Wheeler, A.P. and K.C. Low. 1989. $CaCO_3$ crystal-binding properties of polyanionic proteins and peptides. Abstract, ACS 197th annual meeting.

4. Sikes, C. Steven and A.P. Wheeler. 1990. Surface reactive peptides and polymers: from discovery to commercialization. ACS Books, in preparation.

5. Wheeler, A.P., C.S. Sikes, and K.C. Low. 1988. Absorption of oyster shell organic matrix synthetic analogs to calcium carbonate crystals. Abstract, American Zoologist 57, 133A.

Training Activities: The following students and post-doctoral associates have assisted in the project during year 1.

1.	Donachy, Julie E.	Ph.D.	Research Associate
2.	Dickey, Joan	D.D.S.	Research Associate
3.	Yeung, Miranda	M.S.	Technologist, from Hong Kong
4.	Ding, Jane	B.S.	Surgeon from China working as a technologist
5.	Garris, John	B.S.	beginning M.S. student

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Awards/Fellowships: None.

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